Stem Cell Research 23 (2017) 95–97

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource: Stem Cell Line

Generation of a gene-corrected isogenic control iPSC line from cystic fibrosis patient-specific iPSCs homozygous for p.Phe508del mutation mediated by TALENs and ssODN

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ARTICLE INFO

Article history:
Received 9 February 2017
Received in revised form 13 June 2017
Accepted 7 July 2017
Available online 11 July 2017

ABSTRACT

Cystic fibrosis (CF) is a monogenetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which affects multiple organs. Human induced pluripotent stem cells (iPSCs) derived from CF patients and the generation of isogeneic gene-corrected control cell lines enable disease modelling, drug discovery or toxicological studies and therefore the development of CF patient-specific therapies. We have previously generated a hiPSC line from a CF patient homozygous for the p.Phe508del mutation. Here we used TALENs and single-stranded oligonucleotides to correct the mutated triplet in our CF-iPSC line.

Resource table.

Name of stem cell line	MHHi002-A-1 alternative name: CF[1]iPS5-corr18b13w3C36
Institution	LEBAO, Hannover Medical School
Person who created resource	Sylvia Merkert
Contact person and email	Ulrich Martin; martin.ulrich@mh-hannover.de Sylvia Merkert; merkert.sylvia@mh-hannover.de
Date archived/stock date	November 2014
Origin	Human induced pluripotent stem cell line MHHi002-A, originally derived from endothelial colony forming cells from peripheral blood
Type of resource	Gene-corrected induced pluripotent stem cell line
Sub-type	iPSC line
Key transcription factors	OCT4, SOX2, KLF4, c-MYC (Warlich et al., 2011)
Authentication	Identity and purity confirmed by pluripotency markers, *in vitro* differentiation potential, mutation sequencing and karyotyping (Fig. 1)
Link to related literature	N/A
Information in public databases	N/A
Ethics	The Local Ethics Committee approved the study and informed consent was obtained from the patient.

Resource details

Previously we have generated an induced pluripotent stem cell line (named MHHi002-A) from a male CF patient homozygous for the mutation p.Phe508del in the CFTR gene. Thereby the absence of the three bases CTT results in the deletion of phenylalanine (Phe) in position 508 of the amino acid sequence (Fig. 1A). For the generation of patient-specific iPSCs, endothelial colony forming cells (ECFCs) from the peripheral blood of the CF patient were reprogrammed through overexpression of the codon optimized human pluripotency factors OCT4, SOX2, KLF4 and c-MYC using a 4-in-1 lentiviral vector (Warlich et al., 2011). Here, we used this CF-iPSC line for the correction of the mutated triplet CTT by TALENs and single-stranded oligonucleotides (ssODNs) by applying our established targeting protocol (Merkert et al., 2014). In detail, TALEN plasmids and ssODN were transfected into MHHi002-A cells and by limiting dilution and PCR screening we identified 5 heterozygously targeted clones without any pre-selection. One clone was chosen for further characterization (MHHi002-A-1). Sequence analysis confirmed the mono-allelic correction of the p.Phe508del mutation and karyotype analysis proved chromosomal integrity (Fig. 1C, D). The corrected CF-iPSC line MHHi002-A-1 expresses endogenous core pluripotency markers like OCT4, NANOG, SSEA-4 and TRA-1-60 and could be differentiated *in vitro* into derivatives of all three germ layers (Fig. 1E, F).

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http://dx.doi.org/10.1016/j.scr.2017.07.010

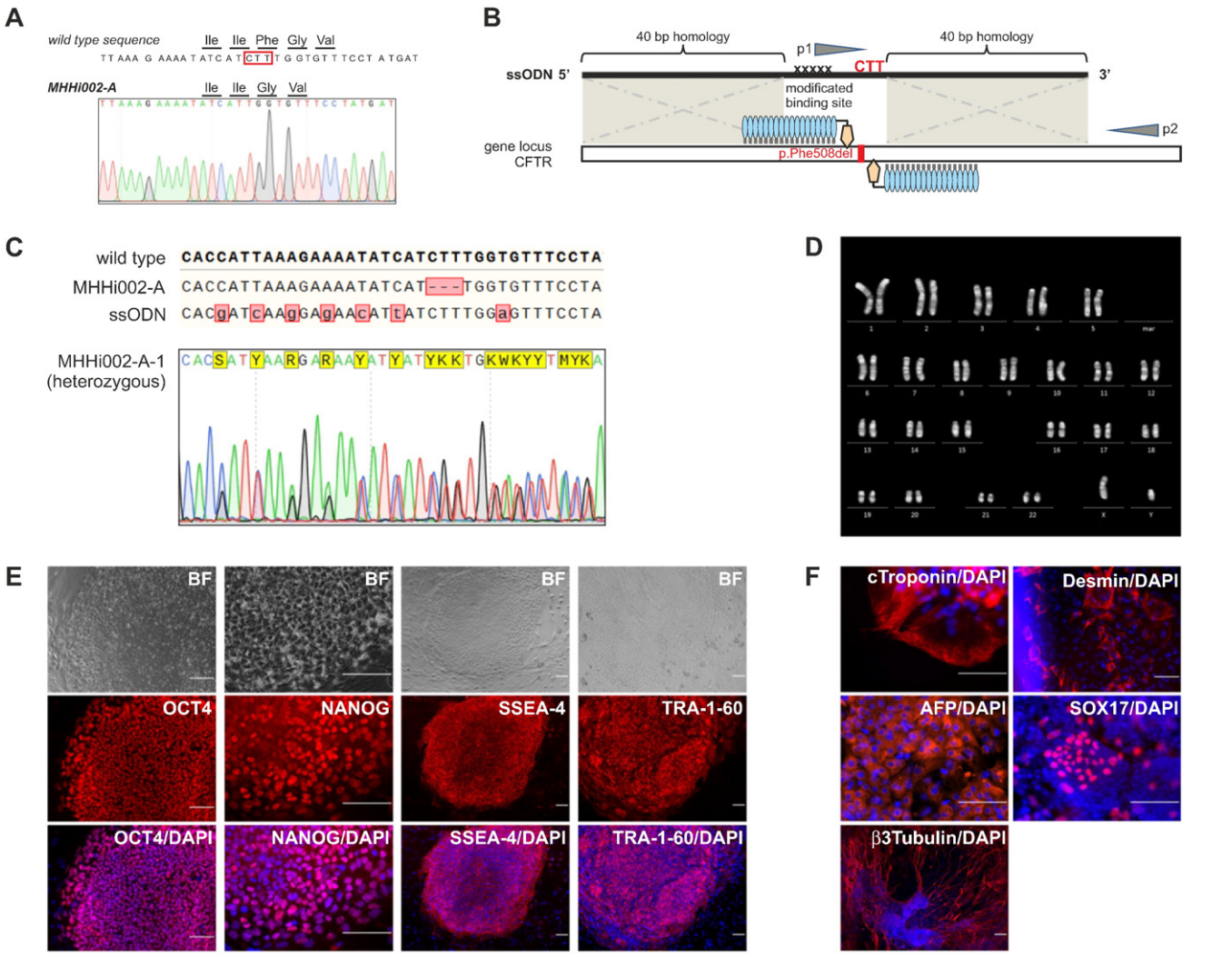

Fig. 1. (A) Sequencing confirms the patient-specific p.Phe508del mutation in MHHi002-A. (B) The schematic shows the 105 bp ssODN donor DNA designed to incorporate the missing CTT into CFTR_ p.Phe508del. Grey arrows indicate screening primers used to confirm the targeted integration of the ssODN. (C) Genotyping of MHHi002-A-1 revealed heterozygous correction of the p.Phe508del mutation. Small letters indicate silent mutations in the applied ssODN, which will result in the wild type amino acid sequence. Yellow frames with the respective single letter code illustrate overlaying sequencing peaks and a DNA frameshift based on the heterozygous alleles. (D) Corrected CF-iPSCs show normal karyotype (46,XY). (E) Immunofluorescence staining for pluripotency markers OCT4, NANOG, SSEA-4 and TRA-1-60 in MHHi002-A-1. Nuclei are stained with DAPI (blue). Scale bars represent 100 μm. BF, bright field. (F) Immunostaining of MHHi002-A-1 derivatives on day 19 of differentiation revealed expression of mesodermal (cTroponin T, Desmin), endodermal (AFP, SOX17) and ectodermal (β3 Tubulin) marker proteins. Nuclei are stained with DAPI (blue). Scale bars represent 100 μm.

Materials and methods

Reprogramming and cell culture conditions

One day prior to transduction, 2×10^5 ECFCs were seeded into a six-well. The adherent cells were transduced with concentrated lentiviral vector pRRL.PPT.SF.hOct34.hKlf4.hSox2.hmyc.i2dTomato.pre (MOI 10) (Warlich et al., 2011) in endothelial culture medium containing 8 μg/ml polybrene (Sigma-Aldrich). The transduced cells were cultured for 6 days in endothelial culture medium without splitting. On day 6, the transduced cells were trypsinised and seeded onto a layer of irradiated murine embryonic fibroblasts (MEFs). On day 7, the culture medium was changed to human ESC medium. Arising iPSC-like colonies were transferred mechanically onto fresh irradiated MEFs and further cultivated clonally.

For feeder cell- based cultures, hiPSCs were cultured on mouse embryonic fibroblasts (MEFs) in knockout-DMEM supplemented with 20% knockout serum replacement, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acid stock (all from ThermoFisher) and 10 ng/ml b-FGF (supplied by the Institute for Technical Chemistry, Leibniz University Hannover, Germany (Chen et al., 2012)). For feeder-free cultures, hiPSCs were expanded as monolayer cultures on Geltrex® (ThermoFisher) as previously described (Burridge et al., 2011) in mTeSR™ medium or MEF-conditioned ESC medium and were harvested by Accutase™ (ThermoFisher). We regularly tested our cell lines for mycoplasma using MycoAlert detection kit (Lonza).

Targeting design

TALENs to target the human CFTR locus (left binding site TGCCTGGCACCATTAAAGA; right binding site GTTTCCTATGATGAATATA) were generated via the golden gate assembly method (Morbitzer et al., 2011). TALENs are based on the NΔ134/C + 17 architecture (Mussolino et al., 2011), contain wild-type *FokI* nuclease domains and the expression is driven by the CMV promoter. The 105 bp ssODN (5′-TTT CAT TCT GTT CTC AGT TTT CCT GGA TTA TGC CTG GCA CgA TcA AgG AgA AcA TtA TCT TTG GaG TTT CCT ATG ATG AAT ATA GAT ACA GAA GCG TCA TCA AAG-3′; small letters indicate silent mutations in the left TALEN binding site) carrying the missing CTT was manufactured by Eurofins MWG Operon (Ebersberg, DE) and was dissolved in water at 100 μM.

Table 1
Primary Antibodies used for immunohistology.

Name	Class	Species	Vendor	Dilition
Anti-OCT4	IgG2b	Mouse	Santa Cruz Biotechnologie, CA, USA	1:100
Anti-NANOG	IgG1	Mouse	Abcam, Cambridge, USA	1:500
Anti-SSEA-4	IgG3	Mouse	Hybridoma Bank, Iowa City, USA	1:100
Anti-TRA-1-60	IgM	Mouse	Abcam, Cambridge, USA	1:100
Anti-Desmin	IgG1	Mouse	Progen, Heidelberg, DE	1:20
Anti-AFP	IgG1	Mouse	R&D Systems, Minneapolis, USA	1:300
Anti-β3 Tubulin	IgG2a	Mouse	Upstate, NY, USA	1:400
Anti-SOX17	IgG	Goat	Millipore, Darmstadt, DE	1:200
Anti-cTroponin T	IgG1	Mouse	Thermo Scientific, St. Leon-Rot, DE	1:100

Transfection

For CFTR correction we adapted our targeting protocol (Merkert et al., 2014) as follows: MHHi002-A cells were expanded as monolayer cultures and transfected with the NEON™ transfection system (ThermoFisher). Therefore, 1×10^6 cells were resuspended in 100 µl NEON™ buffer, electroporated with 5 µg of each TALEN encoding plasmid and 5 µl of the CFTR ssODN and plated onto one Geltrex®-coated 96-well plate with MEF-conditioned ESC medium supplemented with 10 µM ROCK-Inhibitor Y-27632. PCR screening and a second round of limiting dilution revealed single cell clones which were transferred onto MEFs and were further cultivated clonally.

PCR screening and genotyping

For PCR screening cell lysis was done in 96-well plates using DirectPCR Lysis Reagent (PeqLab). Subsequently, 2 µl of the lysate was directly applied for PCR reaction using Phire Hot Start II DNA polymerase (ThermoFisher) according to manufacturer's instructions and following primers: forward 5′-GAT CAA GGA GAA CAT TAT CTT TGG A-3′ and reverse 5′-TAC CCC TCT AAT TCT CTG CTG G-3′. For genotyping, genomic DNA was prepared using QIAamp Blood Mini Kit (QIAGEN) according to manufacturer's instructions and 100 ng of gDNA was amplified by PCR with Phusion® High-Fidelity DNA polymerase (NEB) according to manufacturer's instructions and following primers: forward 5′- GTG CAT AGC AGA GTA CCT GAA ACA G-3′ and reverse 5′-TCA TAG TAA CAT ATT CCC TGC CCT A-3′.The PCR products were sequenced using the forward primer.

Immunocytological staining

Cells were fixed with 4% paraformaldehyde (w/v) and stained by standard protocols using primary antibodies, as listed in Table 1 and secondary antibody (DyLight®549_donkey_anti-mouse_IgG and IgM, DyLight®549_donkey_anti-goat_IgG; 1:200, Jackson Immunoresearch Laboratories). Corresponding isotype antibodies were used for negative control staining. Cells were counterstained with DAPI (Sigma) and analysed with an AxioObserver A1 fluorescence microscope and Axiovision software 4.71 (Zeiss).

In vitro differentiation

HiPSCs were detached from the feeder layer by collagenase IV, dispersed into small clumps and cultured in differentiation medium (80% IMDM supplemented with 20% fetal calf serum, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol and 1% nonessential amino acid stock) in ultra-low attachment plates (Corning Inc., NY, USA) for 7 days. Subsequently, EBs were plated onto 0.1% gelatine coated tissue culture dishes and cultured for a further 13 days before fixation and immunostaining.

Karyotype analysis

After trypsination, metaphases were prepared according to standard procedures. Fluorescence R-banding using chromomycin A3 and methyl green was performed as previously described in detail (Schlegelberger et al., 1999). At least 15 metaphases were analysed per clone at a minimum of 300 bands. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2017.07.010.

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